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Neuromancer Tbx20-related genes (*H15/midline*) promote cell fate specification and morphogenesis of the *Drosophila* heart

Li Qian[a,b], Jiandong Liu[a,b], Rolf Bodmer[a,*]

[a]*The Burnham Institute, Center for Neuroscienes and Aging, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA*
[b]*Graduate Program in Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA*

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Abstract

The Tbx family of transcription factors are prominently expressed in the early cardiac primordium throughout the animal kingdom. Mutations in Tbx genes result invariably in defective formation and function of the heart, including congenital heart disease in humans. Similar to their vertebrate counterpart, the *Drosophila* Tbx20 gene pair, *neuromancer1* (*nmr1*, FlyBase:*H15*) and *neuromancer2* (*nmr2*, Flybase:*mid*), exhibits a dynamic expression pattern, including in all contractile myocardial cells. Deletion mutants of *nmr1* combined with mesoderm-specific knock-down of *nmr2* exhibit phenotypes that suggest *nmr* is critical for correct specification of the cardiac progenitor populations as well as for morphogenesis and assembly of the contractile heart tube. Loss-of-*nmr*-function causes a switch in cell fates in the cardiogenic region, in that the progenitors expressing the homeobox gene *even skipped* (*eve*) are expanded accompanied by a corresponding reduction of the progenitors expressing the homeobox gene *ladybird* (*lbe*). As a result, the number of differentiating myocardial cells is severely reduced whereas pericardial cell populations are expanded. Conversely, pan-mesodermal expression of *nmr* represses *eve*, while causing an expansion of cardiac *lbe* expression, as well as ectopic mesodermal expression of the homeobox gene *tinman*. In addition, *nmr* mutants with less severe penetrance exhibit cell alignment defects of the myocardium at the dorsal midline, suggesting *nmr* is also required for cell polarity acquisition of the heart tube. In exploring the regulation of *nmr*, we find that the GATA factor Pannier is essential for cardiac expression, and acts synergistically with Tinman in promoting *nmr* expression. Moreover, reducing *nmr* function in the absence of *pannier* further aggravates the deficit in cardiac mesoderm specification. Taken together, the data suggest that *nmr* acts both in concert with and subsequent to *pannier* and *tinman* in cardiac specification and differentiation. We propose that *nmr* is another determinant of cardiogenesis, along with *tinman* and *pannier*.

Introduction

The T-box transcription factors are expressed in a wide range of patterns during embryogenesis and play critical roles during many developmental processes, including determination of forelimb/hindlimb identity (Logan and Tabin, 1999; Gibson-Brown et al., 1996), dorsal/ventral patterning in the retina (Koshiba-Takeuchi et al., 2000), specification of endoderm (Xanthos et al., 2001) and mesoderm (Ciruna and Rossant, 2001), and control of proliferation (Hatcher et al., 2001). Mutations in T-box genes are also associated with human congenital diseases, for instance, with the Holt–Oram syndrome (Tbx5; Basson et al., 1997; Li et al., 1997), the DiGeorge syndrome (Tbx1; Jerome and Papaioannou, 2001; Lindsay et al., 2001), the Ulnar Mammary syndrome (Tbx3; Bamshad et al., 1997), with ACTH deficiency (Tbx19; Lamolet et al., 2001) and cleft palate/ankyloglossia (Tbx22; Braybrook et al., 2001). The requirement of Tbx transcription factors in heart development is implicated by a number of striking cardiac defects in mice lacking individual T-box genes including Tbx5, Tbx1, Tbx3 and Tbx6 (Bruneau et al., 2001; Chapman et al., 2003; Hoogaars et al., 2004; Xu et al., 2004). In addition, the prominent cardiac expression of additional T-box genes, such as Tbx2, Tbx18 and Tbx20

* Corresponding author. Fax: +1 858 713 6274.
E-mail address: rolf@burnham.org (R. Bodmer).

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suggest an important role of T-box genes in cardiogenesis (Papaioannou, 2001; Ryan and Chin, 2003).

The role of Tbx20 in heart development is supported by the cardiac expression of Tbx20 in different species. The distinct expression pattern of Tbx20 in the early cardiac progenitor region and the differentiating heart in the fly, zebrafish, *Xenopus*, chick, mouse and human raises the possibility that Tbx20 is a functionally conserved component at the center of the heart-forming process (Brown et al., 2003; Griffin et al., 2000; Kraus et al., 2001; Lio et al., 2001; Meins et al., 2000). This hypothesis is supported by in vitro binding assays showing that mouse Tbx20 can directly interact with the central cardiogenic factors Nkx2-5 and Gata4 and 5 in regulating reporter gene expression (Stennard et al., 2003). Studies in zebrafish using a morpholino-based knockdown approach show a failure of cardiac looping and defects in chamber morphology, suggesting a pivotal role for Tbx20 in heart morphogenesis (Szeto et al., 2002). Here, we use the simple but evolutionary conserved *Drosophila* heart (Bodmer, 1995) as a model to study the role of Tbx20 in cardiac specification and morphogenesis.

The development of the *Drosophila* heart tube begins with the specification of myocardial precursor cells that originate from the dorsal mesoderm and involves the action of the homeobox gene *tinman* and GATA factor encoded by *pannier* (reviewed in Bodmer and Frasch, 1999; Bodmer et al., in press; Zaffran and Frasch, 2002). This initial subdivision of the mesoderm requires additional inductive signal *decapentaplegic (dpp)* and *wingless (wg)* secreted by the overlying dorsal ectoderm to confer competence to the receiving mesoderm cells to form the cardiac primordium (Frasch, 1995; Lockwood and Bodmer, 2002; Lockwood et al., 2001; Park et al., 1996; Wu et al., 1995). As a result, the cells in the dorsal mesoderm are specified into various cardiac progenitors marked by combinatorial patterns of identity gene expression, including the homeobox genes *even skipped* (*eve*) and *ladybird* (*lbe*), and the COUP nuclear hormone receptor gene *seven-up* (*svp*) (Han et al., 2002; Jagla et al., 1997, 2002; Lo and Frasch, 2001; Su et al., 1999). A recent study suggests that specification and positioning of the juxtaposed cardiac cell clusters, marked by Eve and Lbe, require the cooperative action of Hedgehog and Ras signaling (J.L., L.Q. and R.B., unpublished). In the absence of *hh* or *ras*/MAPK activity, the Eve cell clusters are diminished, whereas the Lbe clusters are expanded within the cardiac mesoderm. The stereotyped positioning of Eve and Lbe strongly implicates the involvement of a precise patterning system. Identification of new members in this patterning system is essential to unravel the mechanisms of cardiac specification.

After the cardiac lineages are completed (Alvarez et al., 2003; Han and Bodmer, 2003), the myocardial precursors on either side of the embryo line up in a single row and migrate dorsally to meet their contralateral counterparts at the dorsal midline. Upon contact, the prospective myocardial cells change cell shape during the process of mesenchyme–epithelium transition and acquire their appropriate polarity characteristics (Fremion et al., 1999; Haag and Hartenstein, 1999). This transition and the coordination of correct cell–cell contacts in the forming heart tube are largely unexplored. It is also not known if there is a causal relationship between cardiac cell polarity and patterning or cellular alignment of the forming heart at the dorsal midline.

In *Drosophila*, eight T-box genes have been described (*omb, Byn, org-1, nmr1/H15*, *nmr2/H15r/mid*, *Doc1, Doc2*, *Doc3*; (Buescher et al., 2004; Griffin et al., 2000; Poeck et al., 1993, 1998; Reim et al., 2003; Singer et al., 1996; this work)). Among them, *H15* and the *Doc* triplet show prominent expression in the heart (Griffin et al., 2000; Reim et al., 2003). Recently, the *nmr* (*H15*/*mid*) gene pair has been shown to be a novel negative regulator of *wg* expression, involved in the asymmetric maintenance of *wg* by Hh signaling (Buescher et al., 2004). In the absence of *nmr* function, *wg* is detected not only anterior but also posterior to the Hh stripe causing phenotypes similar to gain-of-*wg*-function. The *Doc* set of T-box genes is involved in dorso-lateral patterning of the epidermis downstream of Dpp and is required for amnioserosa proliferation and differentiation (Reim et al., 2003). Although expressed in the heart, the requirement of *Doc* genes in cardiac development has not yet been elucidated.

In this paper, we investigate the role of the Tbx20 homologs, which we named *neuromancer 1* and *2* (*nmr1 and 2*), in *Drosophila* heart development. Both *nmr* genes are prominently expressed in the forming heart: *nmr2* from late stage 11 on, considerably before *nmr1*. We generated *nmr1* deletion mutants by P-element excision and combined them with *nmr2*-RNAi transgenes as a way of eliminating both Tbx20 gene functions in the mesoderm. These *nmr* double mutants exhibit cardiac specification defects that likely reflect multiple requirements during heart development. In the early heart-forming region of these *nmr* double mutants, the *eve*-expressing clusters are expanded whereas the *lbe*-expressing cardiac progenitors are reduced, without affecting overall cardiac-restricted *tinman*-expression. Due to this misspecification, the lineage (see Han and Bodmer, 2003) and subsequent formation of contractile myocardial cells is severely compromised, as determined by *tinman* and *Dmef2* expression. In moderate *nmr* mutants, a normal number of myocardial cells is generated but they misalign at the dorsal midline during heart tube assembly and exhibit severe polarity defects. Supported also by overexpression and genetic interaction experiments, we conclude that *nmr* encoded T-box genes control multiple phases of cardiac development: (1) specification of the cardiac mesoderm along with *tinman* and *pannier*; (2) distinction between cardiac progenitor populations, namely those expressing *eve* and *lbe*, and (3) epithelial polarity acquisition of the myocardial cells, which seems to be prerequisite for

correct morphogenesis of the heart tube. Together, these data demonstrate the essential roles of Tbx20 genes in cardiac specification and differentiation.

Materials and methods

Fly stocks

The following mutant stocks were used: pnr^{VX6}/TM6-*twi*lacZ (Heitzler et al., 1996; Klinedinst and Bodmer, 2003), tin^{346}/TM3-*ftz*lacZ (Azpiazu and Frasch, 1993), $Dmef2^{P520}$/Cyo-*wg*lacZ (Bour et al., 1995). Overexpression of transgenes was achieved using the UAS-Gal4 system (Brand and Perrimon, 1993). The following lines were used: *twi*-Gal4 (twi>; Greig and Akam, 1993), *24B*-Gal4 (24B>; Brand and Perrimon, 1993), the double combination *twi*-Gal4;*24B*-Gal4 (twi24B>; pan-mesodermal expression, see Lockwood and Bodmer, 2002), *da*-Gal4 (Wodarz et al., 1995), UAS-*eve* (Su et al., 1999), UAS-*pnr* (Haenlin et al., 1997), UAS-*tin* (Ranganayakulu et al., 1998), and the double combination UAS-*pnr*;UAS-*tin* (Klinedinst and Bodmer, 2003).

Imprecise deletion screen for the nmr1 locus

nmr1 alleles $nmr1^{614}$ ($nmr1^{614}$,*b,cn;*ry^{506}), $nmr1^{210}$ ($nmr1^{210}$,*b,cn;*ry^{506}) were generated by imprecise P-element excision using nmr^{H15}-LacZ (*H15[*ry^{+}*],b,cn;* ry^{506}) line, which contains a P-element inserted at −331 bp, 5′ upstream of the *nmr1* transcript start (Brook and Cohen, 1996). *nmr1* deletions were isolated by crossing the nmr^{H15}-LacZ to the transposase-containing line P[Δ2–3] (Robertson et al., 1988; Tsubota and Schedl, 1986). The breakpoints of the deletion mutants were mapped by PCR amplification using the following primer pairs: Forward, AATAGCCAATGAGAAACGGAATGG; Reverse, AGGACTTTTCGTGGGCTCGATTG and GAAAGTAACTGCCAATTGCCGACA. PCR fragments including the breakpoints of $nmr1^{614}$ and $nmr1^{210}$ were sequenced. $nmr1^{614}$ contains a 3,941 bp deletion from −331 to +3610, which includes part of the DNA binding domain (T-box). $nmr1^{210}$ contains a 10,911 bp deletion from −331 bp to +10580 bp, which deletes the entire T-box domain. Both alleles are likely to be null alleles, because with RT-PCR and in situ hybridization no *nmr1* transcripts are detected. Homozygous nmr^{614} and nmr^{210} flies are viable and fertile.

Generation of RNAi knockdown of nmr2

The UAS-*nmr2*RNAi construct was generated using the pWIZ vector (Lee and Carthew, 2003). Briefly, the cDNA corresponding to the coding region 5′ upstream of T-box, which does not have much homology to other sequences in the genome, was amplified by PCR using the following primer pair flanked by an *Xba*I site: Forward, TCTAGAGCGGCCGCTGCAGCACCAAATGCCAGTGG; Reverse, TCTAGAACTGCACCGGCTTCAGATCG. The PCR fragment was subcloned into pGEMT-easy (Promega) and *Xba*I digested. This *Xba*I fragment was sequentially inserted into the *Avr*II and *Nhe*I sites of the pWIZ vector and selected for insertion in the opposite orientation by sequencing. After sequence verification of the construct, P-element mediated transformation yielded 36 independent UAS-*nmr2*RNAi transformant lines.

Overexpression of nmr1 and nmr2

Total RNA extracted from 0 to 16 h old wildtype embryos was used to make cDNA with the First Strand Synthesis kit (Amersham Biosciences). *nmr1* cDNA was amplified by PCR and subcloned into pGEMT-Easy (Promega). The following primer pairs corresponding to the putative 5′ and 3′UTR, respectively, were used: Forward, GTCGTCAAGTCGCAAAGTGTG; Reverse, GGGTGGTTTAGTTGTTTTCGTTGG. Subsequently, the *Eco*RI insert of the full-length *nmr1* cDNA was ligated into the transformation vector pUAST (Brand and Perrimon, 1993). For the generation of UAS-*nmr2*, full-length cDNA derived from the clone RE27439 (Resgen) was digested with *Not*I and *Kpn*I and ligated into the pUAST vector digested with the same enzymes. Several stable transformation lines for both constructs were obtained using standard germline transformation methods.

Immunohistochemistry

Antibody staining, fluorescent in situ hybridization and antibody double labeling were performed as described (Han et al., 2002; Lo et al., 2001). Cy3- or FITC-conjugated secondary antibodies (Jackson Labs) were used for fluorescent confocal microscopy. For Lbe antibody staining, *nmr1* or *nmr2* fluorescent in situ hybridization, the indirect TSA System (Perkin-Elmer) was used to amplify the signal. Embryos with fluorescent staining were mounted in VectaShield (Vector Laboratories) and preparations were analyzed using Zeiss LSM510 and Biorad MRC-1024MP confocal microscopes. The following primary antibodies were used in this study: rabbit anti-Eve, 1:300 (Frasch et al., 1987); mouse anti-Lbe, 1:40 (Jagla et al., 1997); rabbit anti-Tinman, 1:1000 (Venkatesh et al., 2000); rabbit anti-β-Galactosidase, 1:2000 (Cappel); mouse anti-β-Galactosidase, 1:500 (Sigma); rabbit anti-DMef2, 1:2000 (Lilly et al., 1995); rabbit anti-Odd-skipped, 1:200 (Ward and Skeath, 2000); rabbit anti-phospho-H3 (pH3) 1:200 (Upstate Biotechnologies); rabbit anti-Dystroglycan (DG) 1:2000 (Deng et al., 2003). The following primary antibodies used are all from Hybridoma Bank, University of Iowa: mouse anti-Eve, 1:200; mouse anti-Disc large (Dlg) 1:500; mouse anti-Armadillo (Arm) 1:500; mouse anti-α-Spectrin (Spec) 1:100; mouse anti-Wg 1:50; mouse anti-En 1:50. All the secondary antibodies were used at 1:200 (Vector Laborato-

ries). The first and the last exon were used to generate the in situ hybridization probes for *nmr1* and *nmr2*, respectively.

Semi-quantitative RT-PCR analysis

To determine the extent of RNAi knockout of the *nmr2* gene, total RNA was extracted from 0 to 16 h embryos of wildtype, *da*-Gal4/UAS-lacZ, *da*-Gal4/UAS-*nmr2*RNAi using trizol reagent (Promega). Any remaining DNA was removed using DNAase (Invitrogen). First-strand cDNA synthesis was performed using First Strand Synthesis kit for RT-PCR (Amersham Biosciences). Platinum Taq (Invitrogen) was used to perform PCR with *nmr2* primers (Forward: ATCAGAATCAGCTGATCACCAAGCTG. Reverse: ACTGCTGCTGGAACATGTGGAAGG) corresponding to a 360-bp fragment at the 3′ end of *nmr2*. As a control, a 371 bp fragment of the *tubulin* gene was amplified (Forward: CGATGCCAAGAACATGATGG; Reverse: GATCGTTCATGTTGCTCTCG).

Results

Dynamic expression pattern of nmr genes during embryogenesis

Given the documented pattern of vertebrate Tbx20 expression in heart, we wonder whether *Drosophila* Tbx20/*nmr* is also expressed in the forming heart and in what kind of pattern. Using fluorescent in situ hybridization (FISH), we determined that both *nmr1* and *2* genes exhibit similar RNA expression patterns late in embryogenesis, but they differ significantly in the onset of expression (Fig. 1). Cardiac *nmr2* expression initiates at late stage 11 when *tinman* is restricted to the prospective cardiac mesoderm (Fig. 1E; Bodmer et al., 1990), whereas *nmr1* RNA is only detectable after stage 13 (Figs. 1A, B). Both persist in an identical pattern as the bilateral myocardial cells begin to assemble into the heart tube at the dorsal midline (Figs. 1B, C, H, I). *nmr2* is first detected at blastoderm stage as well as later in ventral ectoderm derivatives posterior to Wg and overlapping with En/Hh (Figs. 1J–M).

Since *nmr2* is not expressed in the entire *tinman*-expressing cardiogenic region (Fig. 1E), we conducted fluorescent in situ hybridization of *nmr2* double-labeled with various markers of other set of cardiac progenitor. Indeed, *nmr2* is expressed in a complementary pattern to that of Eve, which marks a subset of pericardial cells (Fig. 1F). After germ band retraction, the *nmr2*-expressing cells differentiate into 6 myocardial cells per hemisegment indicated by double labeling with nmr^{H15}-LacZ (Figs. 1H, I). The exclusive expression of *nmr2* (and *nmr1*) in all myocardial cells that will give rise to the contractile myocardium suggests that the corresponding cardiac progenitors (see Han and Bodmer, 2003) may co-label with Lbe and Svp, in addition to Tinman. Indeed, double-labeling with Lbe shows that they coincide in two myocardial cells per hemisegments (Fig. 1G). Cardiac *nmr* expression (after stage 13) also coincides with other myocardial-specific (e.g., dSUR) but not with pericardial-specific markers (e.g., Zfh-1; data not shown). However, we cannot exclude the possibility that at earlier stages (11/12) mixed or pericardial-only lineages (e.g., expressing *tinman* but not *eve*) transiently express *nmr*.

Generation of loss-of-nmr-function mutants

To determine the requirement for *nmr* in *Drosophila* heart formation, we generated mutants. The *nmr1* mutants, $nmr1^{614}$ and $nmr1^{210}$, were made by imprecise excision of the H15 P-element located 331 bp 5′ to the transcript start (see Materials and methods). In both alleles, the 5′UTR, transcription and translation start and part of T-box domain are deleted (Fig. 2A). In both alleles, no *nmr1* transcripts are detected as determined by RT-PCR and whole-mount in situ hybridization, but *nmr2* RNA appears unchanged (data not shown).

In order to reduce or eliminate *nmr2* function, we took a transgenic snapback RNA interference (RNAi) approach in conjunction with the UAS-Gal4 system (see Materials and methods; Brand and Perrimon, 1993; Lee and Carthew, 2003). The cDNA fragment of *nmr2* with least homology to *nmr1* was used to generate transgenic flies containing UAS-*nmr2*RNAi. When *da*-Gal4 is driving *nmr2*-RNAi ubiquitously, *nmr2* RNA is virtually absent (Fig. 2B). By combining *nmr2*RNAi transgenes with *nmr1* mutants, we generate a quasi-allelic series of loss-of-function for both *nmr* genes. To distinguish the mesodermal versus ectodermal requirement of *nmr*, we generated germlayer-specific *nmr* double mutants. For example, embryos of the genotype $nmr1^{614}$; 24B-Gal4/UAS-*nmr2*RNAi ($nmr1^{-}$,$nmr2^{meso-}$) lack *nmr2* RNA in the heart region (Figs. 2C–E).

Loss of nmr in the mesoderm affects cardiac cell specification

nmr1 mutants are viable and exhibit only mild defects in cardiac morphogenesis (see below), and none of the early heart markers seem to be affected. In twi24B-Gal4/UAS-*nmr2*RNAi ($nmr2^{meso-}$) embryos, however, *eve* expression is moderately expanded (Figs. 3A, B, G). Eliminating both *nmr* genes in the mesoderm ($nmr1^{-}$,$nmr2^{meso-}$), we observe a more severe phenotype, in that at the stage 12, the Eve clusters are dramatically enlarged. Importantly, this expansion appears to be at the expense of *lbe* expression, which is significantly reduced (Figs. 3C–G). Despite the change in the relative proportion of *eve* and *lbe* expression in almost half of the double mutant embryos (Table 1), the extent of cardiac mesoderm delineated by *tinman* expression is not appreciably altered at this stage (Figs. 3C, D). Visceral mesodermal markers also do not seem to be noticeably affected (data not shown).

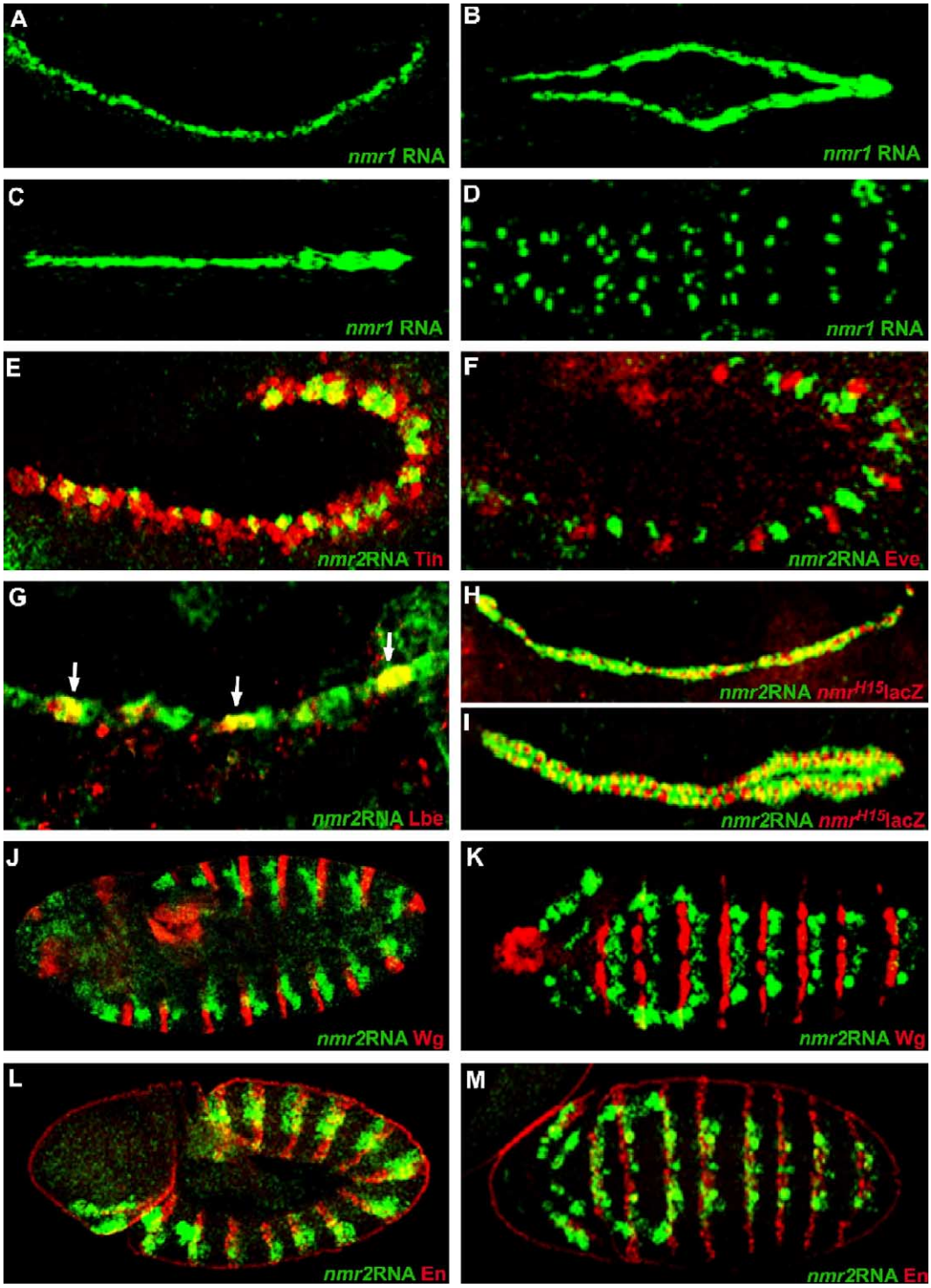

Fig. 1. Dynamic expression pattern of *nmr* genes during *Drosophila* embryogenesis. (A–C) Cardiac *nmr1* transcripts are first detected at stage 13 (A, lateral view) and persist afterwards beyond stage 17 (B, C dorsal view). (D) *nmr1* is also expressed in central nervous system (CNS) (ventral view). (E) Double labeling for *nmr2* RNA (green) and Tinman protein (red) shows *nmr2*-expressing cells are contained within the *tinman*-expressing cardiac mesoderm (lateral view). (F) At stage 12, *nmr2* (green) is expressed in clusters both anterior and posterior to Eve-stained nuclei (red). (G) At stage 14, *nmr2* RNA (green) and Lbe protein (red) co-localize with two prospective myocardial cells (arrows), but not with Lbe-positive pericardial cells (only few are visible). (H, I) After germ band retraction, *nmr2* (green) is expressed in all myocardial cells as is the β-Gal-reporter (red) in nmr^{H15}-LacZ embryos. (J–M) Ectodermal expression of *nmr2* (green) relative to Wingless (Wg) and Engrailed (En) (red). (J, K) *nmr2* transcripts locate posterior to the Wg expression domains (mid-stage 11). (L, M) *nmr2* expression stripes partially overlap with En dorsally (L, stage 10) and abuts posterior to En ventrally (M, stage 13).

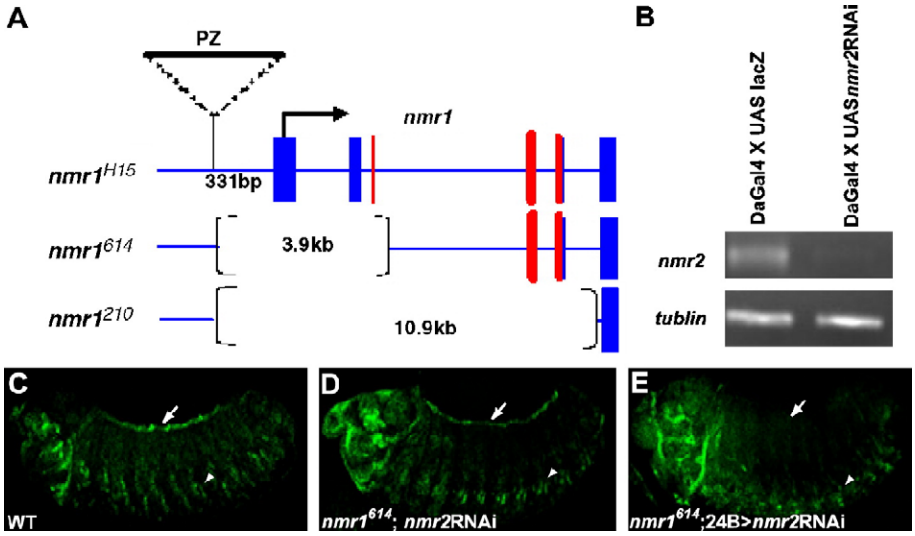

Fig. 2. Generation of *nmr* loss-of-function mutants. (A) Schematic map of *nmr1* deletions. $nmr1^{H15}$ is an enhancer trap line characterized in a screen identifying patterning genes, which shows a prominent reporter gene expression in the embryonic heart (Figs. 1A–C; Brook and Cohen, 1996; Griffin et al., 2000). $nmr1^{614}$ is a 3.9-kb deletion and $nmr1^{210}$ a 10.9-kb deletion. Blue marks the protein-coding region, red the T-box domain. (B) Semi-quantitative RT-PCR for embryos with *da*-Gal4-driven *nmr2*RNAi shows a dramatic reduction in the *nmr2* transcripts compared to *da*-Gal4-driven *lacZ* control. Tubulin is used as a loading control. (C–E) In situ hybridization demonstrates cardiac specific knock-down of *nmr2* RNA. *nmr2* expression in wildtype (C) and $nmr1^{614}$,UAS-*nmr2*RNAi (D) without the mesodermal 24B-Gal4 driver. When UAS-*nmr2*RNAi is specifically targeted to the mesoderm (E), cardiac *nmr2* RNA is virtually absent (arrows) while ectodermal *nmr2* RNA is still present (arrowheads).

Repression of Eve by nmr

Consistent with the increase in Eve cell formation with reduced *nmr* function is the complementary pattern of *nmr2* expression relative to Eve positive cells (Fig. 1F). This raises the possibility that *nmr* normally antagonizes *eve* expression, similarly to the repression of *eve* by *lbe* (Han et al., 2002; Jagla et al., 2002). In order to test this, we ectopically expressed *nmr* to see if this causes a reduction in *eve* expression. Indeed, overexpression of either *nmr* throughout the mesoderm greatly reduces stage 12 *eve* expression while expanding *lbe* (Figs. 3H, I). This repression may not (only) be direct, but may (also) be mediated via activation of *lbe*, for example, which then directly represses *eve* (Han et al., 2002; Jagla et al., 2002). The repressive activity of Nmr is consistent with in vitro mouse cell culture studies, in which Tbx20 acts as a repressor rather than an activator (Plageman and Yutzey, 2004). Interestingly, *eve* is not only repressed by *nmr*, but *eve* itself can repress *nmr* (Figs. 3J, K), again consistent with the cross-repressive relationship between *eve* and *lbe* (Han et al., 2002; Jagla et al., 2002). Therefore, we propose that *nmr* plays a crucial role in the distinction of cardiac cell types during the early events of cardiogenesis.

Misspecification of cardiac cell types in nmr mutants results in less myocardial cells and more pericardial cells

After germ band retraction (stage 13/14), the cardiac progenitor lineages have produced a set of contractile myocardial and pericardial cells that will go on to form the mature heart (Alvarez et al., 2003; Han and Bodmer, 2003). Since at that stage *nmr* expression has become restricted to all prospective myocardial cells, we examined their differentiation in *nmr* mutants, using myocardial- and pericardial-specific markers. Remarkably, even though cardiac-restricted Tinman is not significantly altered at stage 12 (Figs. 3C, D), at stage 14, however, myocardial Tinman is dramatically reduced in $nmr1^{-}$,$nmr2^{meso-}$ embryos (Figs. 4A, B). The remaining Tinman-positive nuclei often co-label with Eve and are thus of pericardial identity (see below). In addition, staining with muscle-specific transcription factor Dmef2, normally present in all myocardial but none of the pericardial nuclei, is less or absent in the heart-forming region in these *nmr* mutant embryos (Figs. 4C, D). We speculate that the reduction in *tinman* and *Dmef2* expression in the forming myocardium is in part due to the loss of *lbe* expression, which includes the Lbe myocardial progenitors co-expressing *tinman*. To account for the loss of non-Lbe positive myocardial cells, we postulate that the Lbe-negative, Tinman-positive myocardial progenitor lineages also require *nmr* function (see Han and Bodmer, 2003, for description of cardiac lineages). Since at stage 12 most if not all cardiac progenitors express *tinman* and their pericardial and myocardial progeny are not yet distinguishable by position, *tinman* expression appears normal. It is only later when the lack of myocardial differentiation becomes apparent (compare Fig. 3D with Fig. 4B).

Even though the non-myocardial Eve progenitor clusters are enlarged in *nmr* mutants, the number of progeny Eve pericardial cells (EPCs) is only moderately increased (Figs. 4B, D, F). We then examined another marker of pericardial cells, Odd-skipped (Odd), which does not overlap with Eve

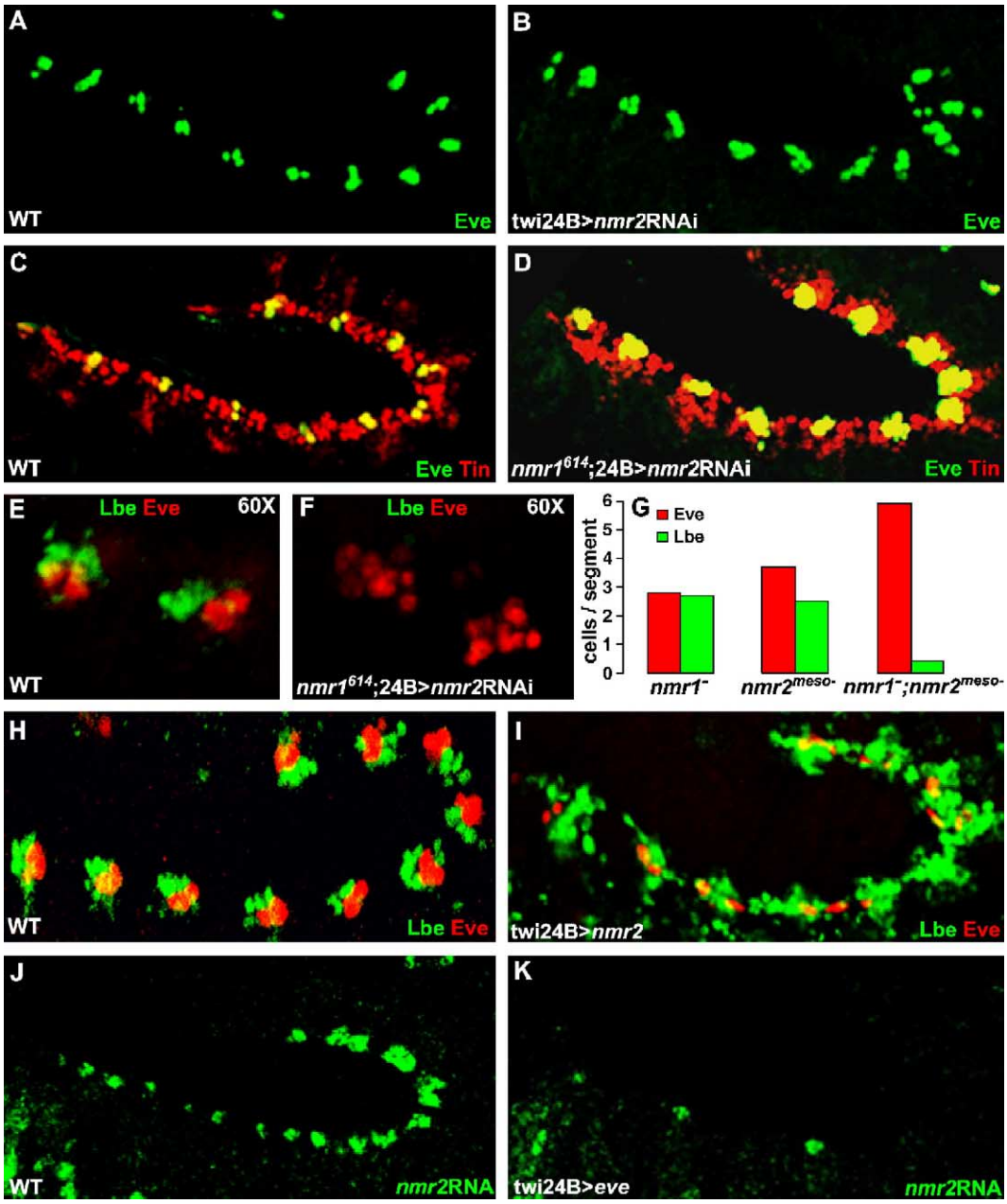

Fig. 3. The requirement of *nmr* in specifying Lbe versus Eve cell fates. (A–F, H–K) Stage 12, (E, D) stage 13 embryos. (A) Wildtype showing the segmental pattern of *eve*-expressing cells. (B) Loss of *nmr2* in the mesoderm by RNAi results in a moderate expansion of Eve cluster. (C, D) Double-labeled embryos for Eve (green) and Tinman (red). (C) Note that *eve*-expressing cells are part of *tinman*-expressing cardiac mesoderm. (D) When UAS-*nmr2*RNAi is targeted in the mesoderm of $nmr1^{614}$ mutants ($nmr1^{-}$,$nmr2^{meso-}$), Eve expression is dramatically expanded to encompass much of the *tinman*-expressing region. (E, F) 60× confocal scans of wildtype (E) and $nmr1^{-}$,$nmr2^{meso-}$ (F) embryo stained for Lbe (green) and Eve (red). Note that $nmr1^{-}$,$nmr2^{meso-}$ results in a complete loss of *lbe* expression, while markedly increasing the number of *eve*-expressing cells. (G) Histogram of average Eve and Lbe cell number per stage 12 hemisegment in $nmr1^{614}$ (Eve: 2.8 ± 0.4 SD n = 18; Lbe 2.7 ± 0.4 SD n = 8), twi24B > *nmr2*RNAi (Eve 3.7 ± 0.8 SD n = 42; Lbe 2.5 ± 0.6 SD n = 35) and $nmr1^{-}$,$nmr2^{meso-}$ (Eve 5.9 ± 2.7 SD n = 21; Lbe 0.4 ± 0.8 SD n = 30). (H) Wildtype embryo double-labeled for Eve and Lbe. (I) When *nmr2* is overexpressed throughout the mesoderm by twi24B-Gal4, Lbe is dramatically expanded at the expense of Eve, which is in sharp contrast to what is observed in *nmr* loss-of-function mutants (D, F, G). (J) Wildtype and (K) *eve* overexpressing embryos labeled for *nmr2* RNA. Note the dramatic reduction of *nmr2* RNA when *eve* is expressed throughout the mesoderm.

(Ward and Skeath, 2000). Indeed, *odd* expression is also expanded in $nmr1^{-}$,$nmr2^{meso-}$ (Figs. 4E, F). This suggests that without *nmr* function pericardial cell populations are expanded, whereas myocardial differentiation is compromised. Conversely, mesodermal overexpression of *nmr* diminishes Eve and Odd pericardial cell fates (Figs. 4G–J), but increases the number of Dmef2 myocardial cells (Figs. 4G, H).

Table 1
Quantification of cardiac phenotypes in *neuromancer* mutants

	Stage 12		Stage 14		Stage 16	
	Eve/Lbe[a]		Reduction in MC[b]		Morphogenesis defects[c]	
	%	*n*	%	*n*	%	*n*
wt	0	24	0	8	0	100
nmr1^{614}	0	21	0	14	5	78
twi24B > *nmr2*RNAi	15	54	0	19	23	62
nmr1^{614};24B > *nmr2*RNAi	44	41	38	34	40	103

Examples of the three *nmr* mutant phenotypes at stage 12, 14 and 16 are given in Figs. 3, 4 and 5 respectively.
[a] Specification defects are assayed at stage 12 by counting embryos with expanded Eve cells at the expense of Lbe cells.
[b] Reduction in myocardial cells (MC) is determined at stage 14 by counting embryos with half the normal Dmef2-expressing cells or less in the heart forming region.
[c] Morphogenesis defects are assayed at stage 16 by counting embryos with a normal number but misaligned Dmef2-expressing myocardial cells at dorsal midline.

nmr induces ectopic tinman expression and cell proliferation

Since *pannier* is able to induce transient ectopic *tinman* expression in the mesoderm (Klinedinst and Bodmer, 2003), we wondered if *nmr* also has that capacity. Indeed, mesodermal expression of either *nmr* gene causes abundant ectopic *tinman* expression until about stage 14 (Figs. 4K, L), but does not persist later (data not shown). This is consistent with the idea that one of *nmr*'s role is to cooperate with *tinman* (and *pannier*, see below) in determining the dorsal-most mesoderm as cardiogenic.

The dramatic *nmr*-induced ectopic *tinman* expression may also be due to increased proliferation of cardiac progenitors. In vertebrates, for example, it has been shown that Tbx5 can inhibit cell proliferation in vitro (Hatcher et al., 2001). Moreover, loss-of-Tbx20-function in mice results in hypoplastic hearts because of reduced cardiac proliferation (S. Evans, personal communication). To determine whether *nmr* affects proliferation in *Drosophila*, we used the mitotic marker phospho-histone H3 (pH3). While wildtype embryos exhibit only few proliferating cells in the dorsal mesoderm at stage 13, pan-mesodermal *nmr* expression increases the number of pH3-labeled nuclei throughout the mesoderm, including the cardiogenic region (Figs. 4M, N). At earlier stages (late 11/12), proliferation is also increased compared to wildtype (data not shown), suggesting that *nmr* is capable of promoting mesoderm proliferation, in addition to its clear role in cardiac lineage specification. Therefore, it is possible that the loss of myocardial cells in *nmr* mutants is not only due to misspecification, but because *nmr* may also be required for myocardial progenitor proliferation. Although the ventral mesoderm also shows excess *nmr*-induced proliferation, it is unlikely, however, that the cells in that region expressing ectopic *tinman* originate from over-proliferating (dorsal) myocardial progenitor, since there is no gradient of ventrally migrating cells observed (Fig. 4L). In order to conclusively address this point, the *nmr* overexpression phenotype will have to be examined in cell cycle arrest mutants.

nmr is required for epithelial polarization of cardiac myocytes

During the process of dorsal closure the bilateral primordia of the heart migrate towards the dorsal midline and acquire a polarity that is the result of a typical mesenchyme–epithelium transition (Fremion et al., 1999). We explored whether the morphogenetic mechanism of coordinate myocardial cell alignment and heart tube assembly is controlled by *nmr*. A large proportion of *nmr2*$^{meso-}$ or *nmr1*$^{-}$,*nmr2*$^{meso-}$ embryos at stage 16/17 exhibit considerable myocardial misalignment defects (Figs. 5A, B; Table 1). This phenotype includes intercalation, gaps, clustering and mis-orientation of the Dmef2-labeled myocardial cells.

To explore the cellular mechanism of how *nmr* regulates myocardial cell alignment, we monitored the cytoskeletal architecture and epithelial polarity features of the forming myocardium. First, we examined the cardiac expression of epithelial polarity markers, namely *discs large* (*dlg*), *armadillo* (*arm*), *crumbs* (*crb*), *dmPar6*, *dystroglycan* (*dg*), *α-spectrin* and *βH-spectrin* (Deng et al., 2003; Fremion et al., 1999; Hurd et al., 2003; Knust and Bossinger, 2002; Petronczki and Knoblich, 2001; Zarnescu and Thomas, 1999). Among them, *dlg*, *arm*, *dg* and *α-spectrin* are expressed in the heart in a stereotyped localized pattern (Figs. 5C, E, G; data not shown).

dlg encodes a MAGUK protein containing three PDZ domains and is required for apical–basal polarity by localizing to the basal–lateral sides of epithelial cells (Bellaiche et al., 2001; Bilder et al., 2003). In myocardial cells of the forming heart tube, however, Dlg protein seems to localize to the dorsal and lateral sides of these cells (Fig. 5C). Even though the leading edge of the dorsally migrating bilateral rows of prospective myocardial cells may be thought of as 'apical', as they meet at the dorsal midline, the 'new' basal side of this forming heart epithelium is facing the midline and prospective lumen of the heart tube, as it is the case for blood vessels in vertebrates (Hogan and Kolodziej, 2002). In any case, Dlg is severely mislocalized in myocardial cells of *nmr* mutants (Figs. 5C, D).

α-Spectrin also marks the basal–lateral membrane of epithelial cells, and in *Drosophila* is detected at the dorsal and lateral sides of the myocardium, similar to Dlg (Fig. 5E). In *nmr* mutants, α-Spectrin is also mislocalized in the forming heart; but unlike Dlg, α-Spectrin levels are much reduced and frequently non-detectable (Fig. 5F). The similar phenotype is observed with Arm (data not shown).

Dg, which is a major component of the Dystrophin–Glycoprotein complex (Michele and Campbell, 2003), plays

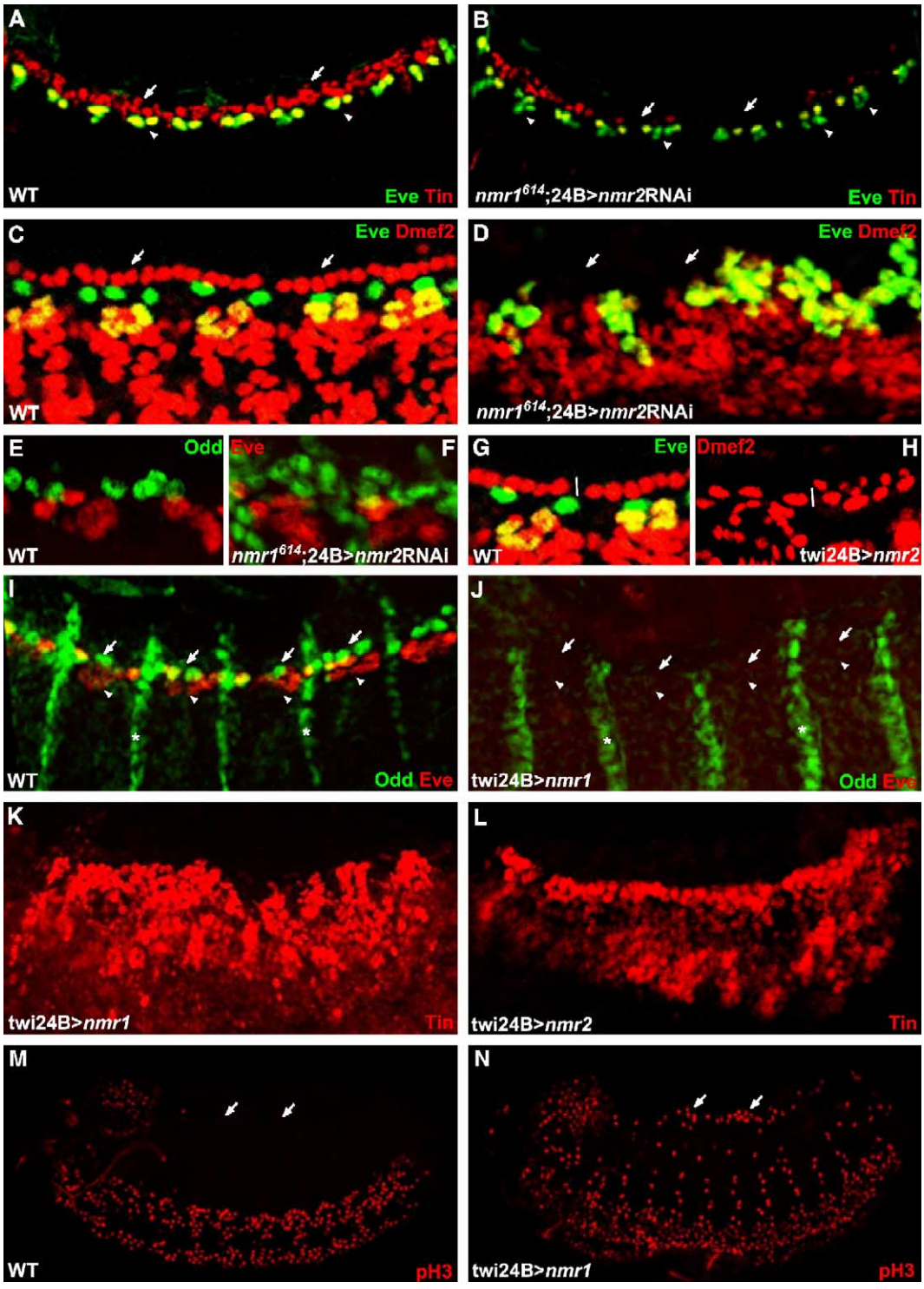

Fig. 4. Myocardial versus pericardial cell fate specification. (A, B) Stage 13 embryos stained for Tinman (red, arrows) and Eve (green, arrowheads) in wildtype (A) and $nmr1^{-},nmr2^{meso-}$ (B) embryos. Note that Tinman in myocardial cells (in red) is reduced in $nmr1^{-},nmr2^{meso-}$ embryos, whereas Tinman co-labeled with Eve (in yellow) is not. (C, D) Stage 15 Dmef2 (red) staining of myocardial cells shows a dramatic reduction (arrows) in $nmr1^{-},nmr2^{meso-}$ (D), as compared to wildtype (C), whereas Eve staining (green) is expanded. (E, F) Stage 13 embryos double-labeled for Odd (green) and Eve (red) exhibit an expansion of Odd and to a lesser extend of Eve (F), compared to wildtype (E). (G, H) Two hemisegments of stage 15 embryos double-labeled for Dmef2 (red) and Eve (green) exhibit an increased number of myocardial cells and much fewer Eve pericardial cells (EPCs) as a result of mesodermal *nmr2* expression (H), compared to wildtype (G). (I, J) Pan-mesodermal expression of *nmr1* completely abolishes both Eve (red, arrowheads) and Odd (green, arrows) expression within the mesoderm (J), as compared to wildtype (I), while ectodermal Odd (asterisk) is unchanged. Overexpression of either *nmr1* (K) or *nmr2* (L) gives arise to ectopic *tinman* expression throughout the mesoderm. (M, N) Cell proliferation is monitored by phospho-histone H3 (pH3) staining in wildtype (M) and *nmr1* overexpressing (N) embryos (stage 13). Note the increase of pH3 labeled nuclei in the cardiogenic region (arrows) when *nmr1* is pan-mesodermally expressed.

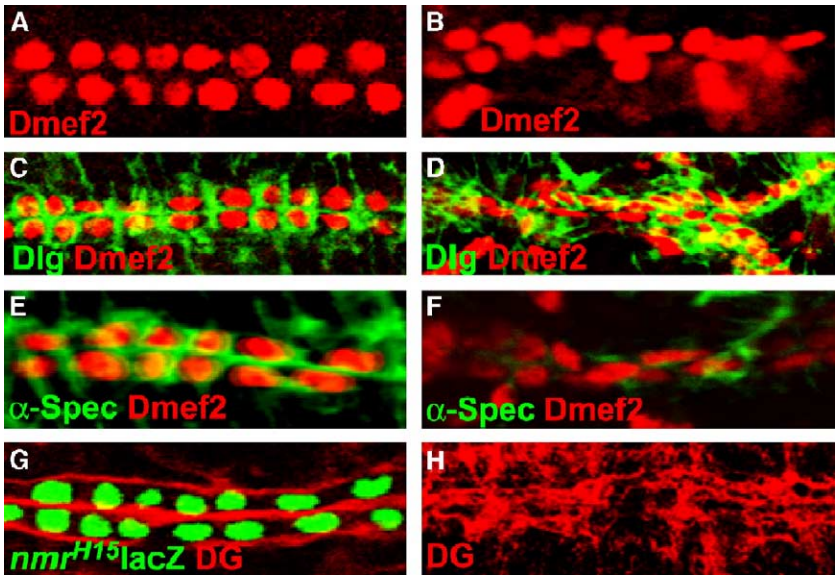

Fig. 5. Heart tube morphogenesis defects in *nmr* mutants. Stage 16 wildtype (A, C, E, G) and *nmr1*$^{-}$,*nmr2*$^{meso-}$ (B, D, F, H) embryos. (A, B) Myocardial cells labeled with Dmef2 are misaligned due to the loss of *nmr* (B), compared to wildtype (A). Note that the number of Dmef2 labeled cells is unchanged. (C) Dlg protein (green) is deposited at the dorsal and lateral sides of wildtype Dmef2-labeled myocardial cells (red). (D) This polarized pattern of Dlg localization is disrupted in *nmr1*$^{-}$,*nmr2*$^{meso-}$ embryos. (E, F) α-Spectrin protein (green), which is normally expressed at the dorsal and lateral sides of myocardial cells (E), is gone or disorganized in the absence of mesodermal *nmr* (F). (G) Double-labeling for Dystroglycan (DG, red) and nuclear *nmr*^{H15}lacZ (green) shows the dorsal and ventral deposition of DG in the myocardial cells (green nuclei) in a wildtype embryo. (H) The organized DG pattern is disrupted in *nmr* mutants.

a crucial role in the establishment of epithelial polarity of the follicle cells in the *Drosophila* ovary (Deng et al., 2003). Dg strongly accumulates at both the basal and apical sides of myocardial membranes, but is excluded laterally (Fig. 5G). As with the other polarity markers, Dg localization is dramatically disrupted in *nmr* mutant embryos (Fig. 5H). The mislocalization of these polarity genes indicates a failure of the myocardium in proper polarity acquisition. Thus, in addition to their role in cell fate specification, the *nmr* encoded Tbx20 genes may also coordinate the mesenchyme–epithelium transition and polarized alignment of the myocardial primordia.

nmr affects myocardial polarity independently of its role in cell fate specification

Since loss-of-*nmr*-function results in early cardiac specification defects, it is possible that the observed abnormalities in morphogenesis are secondary effects to the early defects. Thus, we want to determine whether or not the early misspecifications due to loss-of-*nmr*-function can be separated from the later morphogenesis defects. Instead of expressing *nmr2*RNAi with the pan-mesodermal driver 24B-Gal4, we used the heart-specific tinCΔ4-Gal4 (Lo and Frasch, 2001; Perrin et al., 2004), which drives expression in differentiating myocardial cells as they migrate towards the dorsal midline (Fig. 6A). In tinCΔ4 > *nmr2*RNAi embryos, early cardiac specification do not seem to be affected, based on Tinman, Eve and Dmef2 staining, nor is dorsal migration or alignment of myocardial cells at the dorsal midline (Fig. 6B; data not shown). In contrast, however, epithelial polarity markers are often mislocalized as determined by Dlg, α-Spectrin and Dg staining (Figs. 6C, D; data not shown). Dlg, for example, is no longer detected on the dorsal–lateral sides, but instead is frequently deposited also ventrally (arrows in Fig. 6D) or missing on the dorsal myocardial membranes (arrowheads in Fig. 6D). These data suggest that *nmr* is required for myocardial cell polarization independently of its role in cell fate specification.

Regulation of nmr by pannier

The GATA transcription factor Pannier has previously been shown to be required, along with Tinman, for specification of the heart primordium (Klinedinst and Bodmer, 2003). *pannier* null mutant embryos exhibit a reduction of both myocardial and pericardial cell populations, but *eve* expression is less affected than that of *lbe*. Thus, we wanted to examine the functional relationship between *pannier* and *nmr* in cardiac cell type specification. Double-labeling for *pannier* RNA and *nmr*^{H15}-LacZ shows that reporter gene expression overlaps with that of *pannier* at the dorsal edge of the mesoderm (Fig. 7A). In *pannier* null mutants, mesodermal *nmr* expression is completely missing (Figs. 7B, C), which is in contrast to the presence of residual *tinman* (Figs. 7G, H) and other cardiac marker genes (Figs. 7D, E; Klinedinst and Bodmer, 2003). In addition, pan-mesodermal expression of *pannier* is sufficient to initiate *nmr* expression ectopically (Figs. 7K, L). In contrast, misexpression of *nmr* throughout the mesoderm is unable to induce *pnr* ectopically, and in *nmr1*$^{-}$,*nmr2*$^{meso-}$

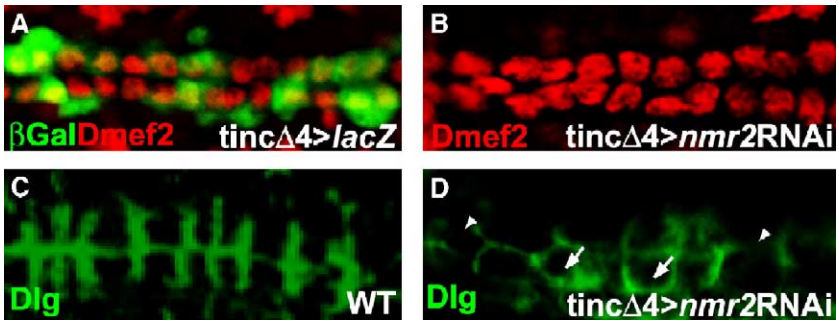

Fig. 6. Disruption in myocardial cell polarity is independent of early specification defects. (A) Dmef2 (red) and LacZ (green) in wildtype embryo with UAS-lacZ driven by tinCΔ4-Gal4 shows the myocardial specificity of this driver. (B) Embryo with UAS-*nmr2*RNAi driven by tinCΔ4-Gal4 (tinCΔ4 > *nmr2*RNAi) shows no detectable myocardial alignment defect, as indicated by Dmef2 staining. (C, D) Dlg is localized at the dorsal and lateral sides of myocardial cells in wildtype (C), but in tinCΔ4 > *nmr2*RNAi embryos (D) Dlg is absent (arrowheads) and/or ectopically localized at the ventral side of the myocardial tube (arrows).

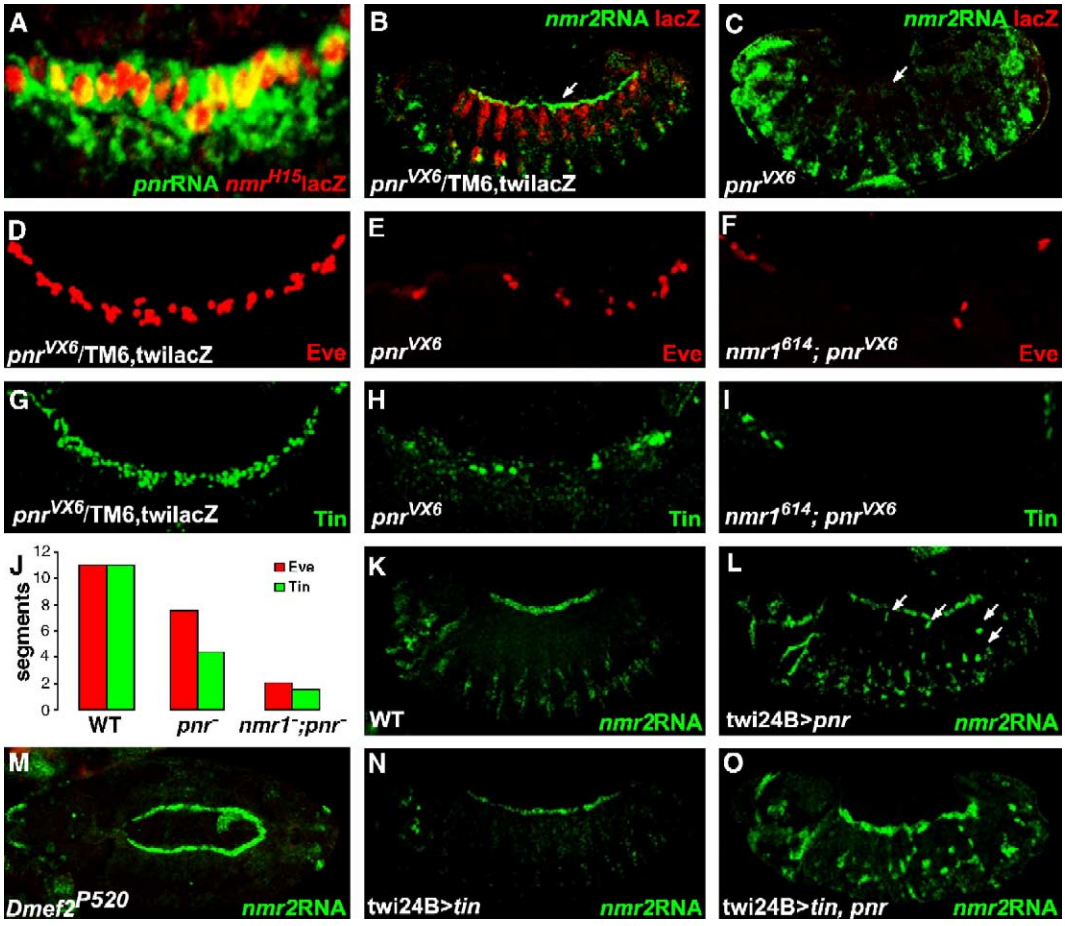

Fig. 7. Regulation of *nmr* by *pannier* and *tinman.* (A) Confocal section through the mesoderm of a nmr^{H15}lacZ (red) embryo at stage 13/14 co-labeled for *pnr* RNA (green). Note that *pnr* RNA encircles LacZ-labeled myocardial nuclei. (B, C) *nmr2* expression (green) in the cardiac region (arrow) is abolished in *pnr* null mutants (C), as compared to its heterozygous sibling controls marked with lacZ (red; TM6-*twi*-lacZ). Eve (D–F) and Tinman (Tin) (G–I) protein are reduced in homozygous pnr^{VX6} mutants (E, H), and virtually absent in $nmr1^{614}$;pnr^{VX6} double mutants (F, I), as compared to pnr^{VX6} heterozygotes (D, G). (J) Histogram of the phenotypes observed in (D–I). Note the synergistic enhancement of the *pannier* phenotype by *nmr* in $nmr1^{614}$;pnr^{VX6} double mutants. Wildtype (WT): 11 Eve-positive hemisegments per embryo side (Eve h/e) (number of half embryos counted, $n = 18$), 11 Tinman-positive h/e (Tin h/e) ($n = 12$); pnr^{VX6}: 7.6 Eve h/e ($n = 21$), 4.4 Tin h/e ($n = 19$), $nmr1^{614}$;pnr^{VX6}: 2.1 Eve-h/e ($n = 17$); 1.6 Tin-h/e ($n = 23$). (K, L) Overexpression of *pnr* with twi24B-Gal4 induces ectopic *nmr2* expression within the mesoderm (L, arrows), as compared to wildtype (K). (M) In a Dmef2 null mutant, $Dmef2^{P520}$, *nmr2* expression is not affected. (N) Pan-mesodermal expression of *tinman* is unable to induce ectopic *nmr2* expression, as compared to wildtype (K). Ectopic *nmr2* induction by mesodermal *pannier* overexpression (L) is synergistically enhanced in the presence of co-expression of *tinman* (O).

embryos no detectable change in *pannier* expression is observed (data not shown). These findings are consistent with the idea that *nmr* acts downstream of *pannier*.

To further examine the relationship between *nmr* and *pannier*, we examined whether they interact genetically. In *pannier* mutants, the Eve clusters are moderately reduced (Figs. 7D, E, J), whereas in *nmr* mutants an increase in the size of these clusters is observed (Figs. 3B, D). In contrast, a further Eve cluster reduction is observed in *nmr1;pnr* double mutants (Figs. 7F, J). A similar synergistic enhancement of the *pannier* phenotype by *nmr1* is observed using Tinman as a marker (Figs. 7G–I). This is consistent with in vitro data that show a direct interaction between Tbx and Gata factors (Garg et al., 2003; Stennard et al., 2003). However, this also raises the question why $nmr1^{-}$ interacts synergistically with $nmr2^{meso-}$ to enlarge the Eve clusters (Fig. 3G), whereas with $pannier^{-}$ a further reduction of Eve is observed. It is thus possible that during cardiac mesoderm specification *nmr* not only plays an essential role in the distinction between Eve and Lbe progenitors, but also acts (redundantly) in conjunction with *pannier* to maintain *tinman* expression, which in turn is required for maintaining *eve* expression. In agreement with this interpretation is the observation that *nmr* overexpression can induce *tinman* ectopically.

We also investigated whether *nmr* expression is regulated by additional cardiac transcription factors. As expected, in *tinman* null mutants no cardiac *nmr* expression is observed (data not shown), since the cardiogenic region is not specified (Bodmer, 1993). Mesodermal overexpression of *tinman*, however, is unable to induce ectopic *nmr* expression suggesting other factors may cooperate together with *tinman* to initiate *nmr* expression (Fig. 7N). Indeed, when we overexpressed *tinman* together with *pannier* throughout the mesoderm, we observed significantly more ectopic *nmr* expression than with *pannier* alone (Figs. 7L, O), suggesting that *tinman* and *pannier* act synergistically to induce *nmr* and other cardiac marker gene expression (see also Klinedinst and Bodmer, 2003). We also examined *Dmef2* mutants, in which muscle myosin fails to be expressed (Bour et al., 1995; Black and Olson, 1998), and found that *nmr* is expressed normally (Fig. 7M), consistent with *Dmef2*'s role in myocardial differentiation rather than in specification of cardiac progenitors.

Discussion

The two closely related *Drosophila* T-box genes, *nmr1* and *nmr2*, have high homology to the single Tbx20 gene in vertebrates (Meins et al., 2000; Reim et al., 2003). They are located in close proximity in the genome and exhibit striking similarities in expression, suggesting they have arisen by a recent duplication. This is further supported by the observed redundancy in function, in that single mutants of either gene have weaker or no phenotype, as compared to double mutants (Table 1; see also Buescher et al., 2004). During cardiac induction, *nmr2* expression overlaps extensively with the cardiac determinants, *tinman* and *pannier*, in the heart-forming region. Cardiac induction of *nmr2* strongly depends on *pannier* and *tinman* function but not vice versa. By mesoderm-specific expression of a transgenic *nmr2*RNAi construct in an *nmr1* mutant background ($nmr1^{-},nmr2^{meso-}$), we show that *nmr* function is essential for both cardiac specification as well as for myocardial polarity acquisition.

Function of nmr in cardiac specification

In $nmr1^{-},nmr2^{meso-}$ embryos, one subpopulation of cardiac progenitors, the Eve cluster, is expanded within the cardiogenic region while another set of cardiac progenitors, the Lbe cluster, is reduced. In addition, *nmr* genetically interacts with *pannier* by synergistically aggravating *pannier*'s cardiac phenotype. These findings in conjunction with the timing and pattern of expression suggest that *nmr* participates, along with *tinman* and *pannier*, in the initial specification and cell type allocation within the heart-forming mesoderm.

The switch in cardiac cell type specification due to loss-of-*nmr*-function is reminiscent of the phenotype observed in embryos with increased Hedgehog or Ras signaling, in which *lbe* expression is eliminated while *eve* is greatly expanded within the cardiogenic mesoderm (J.L, L.Q. and R.B., unpubl.). Hedgehog signaling seems to regulate the expression level of the EGF receptor ligand protease, encoded by *rhomboid* (*rho*), thus activating Ras signal transduction. This raises the possibility that Nmr may act as a link between ectodermal Hedgehog signaling and cardiac patterning. It would be interesting to find out whether Hedgehog signaling limits the expression or activity of mesodermal *nmr*, which in turn might antagonize Ras signaling to prevent formation of Eve-expressing progenitors. Interestingly, not only is *eve* expression abolished upon *nmr* overexpression, but ectopic Eve can also suppress *nmr* expression, suggesting that there is a reciprocal antagonism between *nmr* and *eve* (Fig. 8), as between *lbe* and *eve* (Han et al., 2002; Jagla et al., 2002). It will be interesting to find out if *nmr* expression is expanded in *eve* mutants, in which mesodermal *eve* expression is selectively eliminated (Han et al., 2002).

The initially expanded size of the Eve progenitor clusters in $nmr1^{-},nmr2^{meso-}$ embryos does not persist to the same extent after the germ band has retracted and the EPCs and muscle founder cells have begun to differentiate. It is possible that from the postulated pre-muscular Eve cluster (Carmena et al., 1995, 1998), although initially enlarged in *nmr* mutants, a normal number of progenitors are selected. Alternatively, there may be fewer cell divisions by the Eve progenitors, consistent with a possible role of Tbx20 in proliferation. In contrast, another type of emerging pericardial cells, marked by *odd* expression,

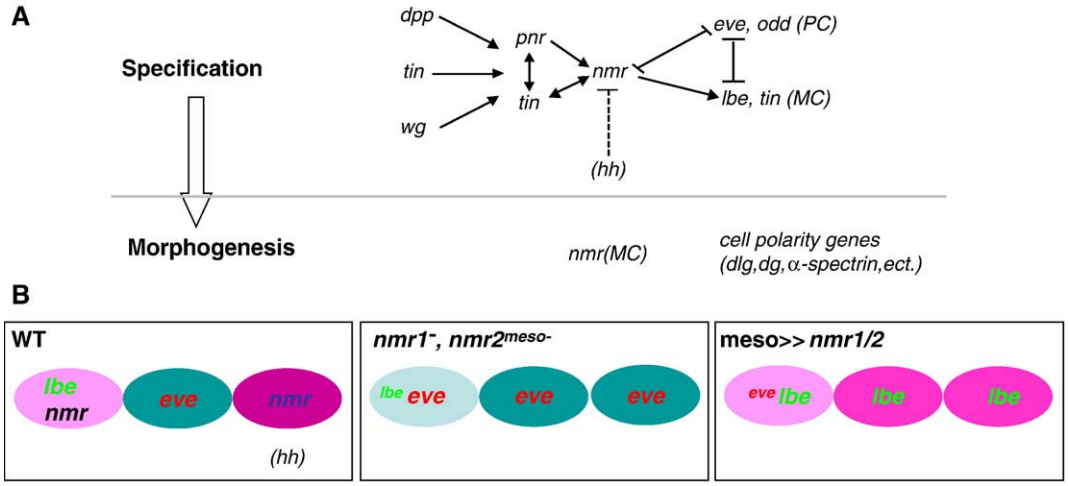

Fig. 8. Model for T-box *nmr* function in the gene network of heart development. (A) *pannier* (*pnr*) and *tinman* (*tin*) expression in the cardiogenic region at the dorsal mesodermal margin is initiated by ectodermal Wg and Dpp signaling in the context of broadly expressed mesodermal *tinman*. Our data suggest that *nmr* might have a dual role: early, in early cardiac specification, and later, in morphogenesis of the heart tube. Early, *nmr* acts together with *pannier* and *tinman* to specify the cardiogenic mesoderm. In addition, *nmr* functions in distinguishing subpopulations of cardiac progenitors, such as the pericardial (PC) Eve and Odd progenitors versus the myocardial (MC) Lbe and Tin progenitors (*eve* and *lbe* also mutually repress each other). It is suggested that *nmr* negatively influences the positional information provided by ectodermal Hedgehog (Hh) signaling (J.L., L.Q. and R.B., unpubl.). Later, after cell type specification, the bilateral cardiac cells migrate towards the dorsal midline, where *nmr* seems to control myocardial cell alignment and polarization. (B) Illustration of the spatial relationship along the anterior–posterior axis between *nmr*, *eve* and *lbe* expressing cells as they become specified at late stage 11/12. In wild type, *nmr2* is expressed both anterior and posterior to Eve (Fig. 1F), while Lbe is present in a cell cluster immediately anterior to that of Eve (Fig. 3H). In the absence of *nmr* activity, Eve is expanded at the expense of Lbe, while pan-mesodermal *nmr* expression diminishes Eve and expands Lbe.

remains expanded. These progenitors emerge at a later time than the Eve cells (Ward and Skeath, 2000) and are possibly subject to a different mechanism of selection and differentiation.

Function of nmr in heart tube morphogenesis

The processes of cardiac morphogenesis are rather complex and not very well understood beyond a descriptive level. Of them, the *Drosophila* heart is remarkably simple but nevertheless resembles that of vertebrates in its initial assembly from bilateral primordial tissue into a highly organized linear vessel-like tube (Bodmer, 1995; Bodmer and Frasch, 1999). It is thus of interest to understand the control mechanisms by which a primitive heart tube forms. The defects in myocardial alignment, heart tube assembly and proper polarity acquisition we observe in *nmr* mutants indicate that these T-box genes play a crucial role during these processes of cardiac morphogenesis. Moreover, when *nmr2*RNAi is specifically targeted to the differentiating myocardial cells, the overall alignment of the bilateral myocardial primordia at the dorsal midline is little affected but the correct epithelial polarity is not established. Together, these observations further suggest that cardiac determination and distinction of cell types are controlled by mechanisms that are separable and perhaps independent from those that govern heart tube assembly and cellular polarity prerequisite for morphogenesis. It will be interesting to find out what transcriptional targets *nmr* controls at progressively later stages of heart development, and whether vertebrate Tbx20 also influence multiple steps during heart development.

In this report, we provide first evidence that *nmr*/Tbx20 also has a role in regulating myocardial polarization, in addition to its role in cardiac cell specification. Studies of cell polarity in other tissues, such as salivary gland, oocyte, neuroblast, suggest cell polarization is important for organelle transport and cell–cell communication via adherens junctions or extracellular matrix components (Deng et al., 2003; Hurd et al., 2003; Myat and Andrew, 2002; Petronczki and Knoblich, 2001). Thus, a failure in proper polarity acquisition is likely to contribute to the observed disorganization of the heart tube. Since only some of the polarity markers we tested exhibit a typical epithelial subcellular distribution in the forming myocardium, we speculate that there may be significant differences by which the heart epithelium is involved in this organ's morphogenesis. Further explorations into the process of cardiac cell polarization are necessary to obtain a mechanistic understanding of cardiac morphogenesis.

The role of nmr in the regulatory network of Drosophila heart development

Our genetic analysis demonstrates a regulatory interaction between *nmr* and other pivotal factors involved in *Drosophila* heart development, namely *pannier* and *tinman*. For example, loss-of-*pannier*-function abolishes mesodermal *nmr* expression, while pan-mesodermal overexpression of *pannier* results in ectopic *nmr* expression, which is

synergistically augmented by co-expression of *tinman*. This is consistent with the idea that *nmr* is a transcriptional target of the cardiac determinants, Pannier and Tinman. Interestingly, the emerging cardiac cell types exhibit a differential susceptibility to *pannier*; pericardial Eve being the least sensitive, which is consistent with the absence of GATA consensus binding sites in the mesodermal *eve* enhancer (Han et al., 2002; Klinedinst and Bodmer, 2003; data not shown).

nmr does not only appear to be a target of *pannier* but also interacts with *pannier* genetically in the overall formation of cardiac mesoderm: *nmr1;pnr* double mutants have a more dramatic reduction in heart-associated cells stained with Tinman or Eve than *pannier* single mutants (*nmr1* single mutants do not exhibit a cell specification phenotype). This indicates that *nmr* also participates in the initial specification of cardiac competence, but this particular role of *nmr* is redundant in the presence of *pannier*, since $nmr1^{-}$,$nmr2^{meso-}$ embryos initially have a normal level of cardiac-restricted *tinman* expression. This idea is supported by the finding that overexpression of *nmr* is sufficient to drive ectopic *tinman* expression. Thus, it appears that *nmr* has two, in part opposing, roles during early cardiac specification: it acts redundantly with *pannier* to specify *tinman*-expressing cardiac mesoderm from which all cardiac cell types emerge, but also acts in distinguishing subpopulations of cardiac progenitors (Lbe versus Eve cell fates, see above), perhaps by negatively mediating positional information provided by the ectodermal Hedgehog signal (J.L., L.Q. and R.B., unpubl.).

Does Drosophila nmr function similarly to its vertebrate homologue Tbx20?

Tbx20 exhibits prominent expression in the heart of all vertebrate and invertebrate organisms examined, and phylogenetic analysis places Tbx20 within the Tbx1 subfamily that is distinct of Tbx5, suggesting evolutionary conservation (Meins et al., 2000; Reim et al., 2003). In Zebrafish, *Tbx20/hrT* is co-expressed with *Nkx2-5* and *Gata4* in cardiogenic as well as in non-cardiac mesoderm adjacent to the tail bud (Szeto et al., 2002). Since we observe a genetic interaction in *Drosophila* between *Tbx20/nmr* and *Nkx2-5/tinman*, *Gata4/pannier*, it may be that a similar interaction takes place in vertebrates. Morpholino knockdown of *Tbx20/hrT* in zebrafish apparently affects chamber morphology, which is consistent with our observations of morphogenesis defects in *Drosophila nmr* mutants. Whether Tbx20 plays a role in cardiac cell polarization in vertebrates remains to be determined. In chicken, Tbx20 is expressed in a complementary pattern to Tbx5 in the embryonic ventricles, and in ovo electroporation and cell culture studies suggest an antagonistic relationship between them (Plageman et al., 2004; Takeuchi et al., 2003). This is reminiscent of the complementary expression and antagonism observed between *nmr* and *eve* in flies, which lack a Tbx5 homologue. Taken together, our studies suggest that Tbx20 genes are likely to play multiple roles during cardiogenesis, ranging from specification, proliferation and morphogenesis (this work; S. Evans, unpublished) to cardiac physiology (L.Q. and R.B., unpublished).

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Note added in proof

Similar findings with regard to the cardioblast alignment phenotype are also reported by Miskolczi-McCallum et al. (Dev. Biol., 2005).

References

Alvarez, A.D., Shi, W., Wilson, B.A., Skeath, J.B., 2003. Pannier and pointedP2 act sequentially to regulate *Drosophila* heart development. Development 130, 3015–3026.

Azpiazu, N., Frasch, M., 1993. *Tinman and bagpipe*: two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. Genes Dev. 7, 1325–1340.

Bamshad, M., Lin, R.C., Law, D.J., Watkins, W.C., Krakowiak, P.A., Moore, M.E., Franceschini, P., Lala, R., Holmes, L.B., Gebuhr, T.C., 1997. Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. Nat. Genet. 16, 311–315.

Basson, C.T., Bachinsky, D.R., Lin, R.C., Levi, T., Elkins, J.A., Soults, J., Grayzel, D., Kroumpouzou, E., Traill, T.A., Leblanc-Straceski, J., Renault, B., Kucherlapati, R., Seidman, J.G., Seidman, C.E., 1997. Mutations in human TBX5 cause limb and cardiac malformation in Holt–Oram syndrome. Nat. Genet. 15, 30–35.

Bellaiche, Y., Radovic, A., Woods, D.F., Hough, C.D., Parmentier, M., O'Kane, C.J., Bryant, P.J., Schweisguth, F., 2001. The partner of inscuteable/discs-large complex is required to establish planar polarity during asymmetric cell division in *Drosophila*. Cell 106, 355–366.

Bilder, D., Schober, M., Perrimon, N., 2003. Integrated activity of PDZ protein complexes regulates epithelial polarity. Nat. Cell Biol. 5, 53–58.

Bodmer, R., 1993. The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. Development 118, 719–729.

Bodmer, R., 1995. Heart development in *Drosophila* and its relationship to vertebrate systems. Trends Cardiovasc. Med. 5, 21–27.

Bodmer, R., Frasch, M., 1999. Genetic determination of *Drosophila* heart development. In: Rosenthal, N., Harvey, R. (Eds.), Heart development. Academic Press, San Diego, pp. 65–90.

Bodmer, R., Jan, L.Y., Jan, Y.N., 1990. A new homeobox-containing gene, msh-2, is transiently expressed early during mesoderm formation of *Drosophila*. Development 110, 661–669.

Bodmer, R., Wessells, R.J., Johnson, E.C., Dowse, H., 2004. Heart

development and function. In Comprehensive molecular insect science, vol. 2. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), volumes 1–7, Elsevier, in press.

Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., Taghert, P.H., Abmayr, S.M., Nguyen, H.T., 1995. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. Genes Dev. 9, 730–741.

Black, B.L., Olson, E.N., 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu. Rev. Cell Dev. Biol. 14, 167–196.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Braybrook, C., Doudney, K., Marcano, A.C., Arnason, A., Bjornsson, A., Patton, M.A., Goodfellow, P.J., Moore, G.E., Stanier, P., 2001. The T-box transcription factor gene TBX22 is mutated in X-linked cleft palate and ankyloglossia. Nat. Genet. 29, 179–183.

Brook, W.J., Cohen, S.M., 1996. Antagonistic interactions between wingless and decapentaplegic responsible for dorsal–ventral pattern in the *Drosophila* Leg. Science 273 (5280), 1373–1377.

Brown, D.D., Binder, O., Pagratis, M., Parr, B.A., Conlon, F.L., 2003. Developmental expression of the *Xenopus laevis* Tbx20 orthologue. Dev. Genes Evol. 212, 604–607.

Bruneau, B.G., Nemer, G., Schmitt, J.P., Charron, F., Robitaille, L., Caron, S., Conner, D.A., Gessler, M., Nemer, M., Seidman, C.E., Seidman, J.G., 2001. A murine model of Holt–Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell 106, 709–721.

Buescher, M., Svendsen, P.C., Tio, M., Miskolczi-McCallum, C., Tear, G., Brook, W.J., Chia, W., 2004. *Drosophila* T box proteins break the symmetry of hedgehog-dependent activation of wingless. Curr. Biol. 14, 1694–1702.

Carmena, A., Bate, M., Jimenez, F., 1995. Lethal of scute, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. Genes Dev. 9, 2373–2383.

Carmena, A., Gisselbrecht, S., Harrison, J., Jimenez, F., Michelson, A.M., 1998. Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. Genes Dev. 15, 3910–3922.

Ciruna, B., Rossant, J., 2001. FGF signaling regulates mesodermal cell fate specification and morphogenetic movement at the primitive streak. Dev. Cell 1, 37–49.

Chapman, D.L., Cooper-Morgan, A., Harrelson, Z., Papaioannou, V.E., 2003. Critical role for Tbx6 in mesoderm specification in the mouse embryo. Mech. Dev. 120, 837–847.

Deng, W.M., Schneider, M., Frock, R., Castillejo-Lopez, C., Gaman, E.A., Baumgartner, S., Ruohola-Baker, H., 2003. Dystroglycan is required for polarizing the epithelial cells and the oocyte in *Drosophila*. Development 130, 173–184.

Frasch, M., 1995. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. Nature 374, 464–467.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H., Levine, M., 1987. Characterization and localization of the even-skipped protein of *Drosophila*. EMBO J. 6, 749–759.

Fremion, F., Astier, M., Zaffran, S., Guillen, A., Homburger, V., Semeriva, M., 1999. The heterotrimeric protein Go is required for the formation of heart epithelium in *Drosophila*. J. Cell Biol. 145, 1063–1076.

Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J.C., Srivastava, D., 2003. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 424, 443–447.

Gibson-Brown, J.J., Agulnik, S.I., Chapman, D.L., Alexiou, M., Garvey, N., Silver, L.M., Papaioannou, V.E., 1996. Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. Mech. Dev. 56, 93–101.

Greig, S., Akam, M., 1993. Homeotic genes autonomously specify one aspect of pattern in the *Drosophila* mesoderm. Nature 362, 630–635.

Griffin, K.J.P., Stoller, J., Gibson, M., Chen, S., Yelon, D., Stainier, D.Y.R., Kimelman, D., 2000. A conserved role for H15-related T-box transcription factors in zebrafish and *Drosophila* heart formation. Dev. Biol. 218, 235–247.

Haag, T.A., Hartenstein, V., 1999. The role of cell adhesion molecules in *Drosophila* heart morphogenesis: faint sausage, shortgun/DE-cadherin, and laminin A are required for discrete stages in heart development. Dev. Biol. 208, 56–69.

Haenlin, M., Cubadda, Y., Blondeau, F., Heitzler, P., Lutz, Y., Simpson, P., Ramain, P., 1997. Transcriptional activity of pannier is regulated negatively by heterodimerization of the GATA DNA-binding domain with a cofactor encoded by the u-shaped gene of *Drosophila*. Genes Dev. 11, 3096–3108.

Han, Z., Bodmer, R., 2003. Myogenic cells fates are antagonized by Notch only in asymmetric lineages of the *Drosophila* heart, with or without cell division. Development 130, 3039–3051.

Han, Z., Fujioka, M., Su, M., Liu, M., Jaynes, J.B., Bodmer, R., 2002. Transcriptional integration of competence modulated by mutual repression generates cell-type specificity within the cardiogenic mesoderm. Dev. Biol. 252, 225–240.

Hatcher, C.J., Kim, M.-S., Mah, C.S., Goldstein, M.M., Wong, B., Mikawa, T., Basson, C.T., 2001. TBX5 transcription factor regulates cell proliferation during cardiogenesis. Dev. Biol. 230, 177–188.

Heitzler, P., Haenlin, M., Ramain, P., Calleja, M., Simpson, P., 1996. A genetic analysis of pannier, a gene necessary for viability of dorsal tissues and bristle positioning in *Drosophila*. Genetics 143, 1271–1286.

Hogan, B.M., Kolodziej, P.A., 2002. Molecular mechanism of tubulogenesis. Nat. Genet. 3, 513–523.

Hoogaars, W.M., Tessari, A., Moorman, A.F., de Boer, P.A., Hagoort, J., Soufan, A.T., Campione, M., Christoffels, V.M., 2004. The transcriptional repressor Tbx3 delineates the developing central conduction system of the heart. Cardiovasc. Res. 62, 489–499.

Hurd, T.W., Gao, L., Roh, M.H., Macara, I.G., Margolis, B., 2003. Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. Nat. Cell Biol. 5, 137–142.

Jagla, K., Frasch, M., Jagla, T., Dretzen, G., Bellard, F., Bellard, M., 1997. *Ladybird*, a new component of the cardiogenic pathway in *Drosophila* required for diversification of heart precursors. Development 124, 3471–3479.

Jagla, T., Bidet, Y., Da Ponte, J.P., Dastugue, B., Jagla, J., 2002. Cross-repression interactions of identity genes are essential for proper specification of cardiac and muscular fates in *Drosophila*. Development 129, 1037–1047.

Jerome, L.A., Papaioannou, V.E., 2001. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. Nat. Genet. 27, 286–291.

Klinedinst, S.L., Bodmer, R., 2003. Gata factor Pannier is required to establish competence for heart progenitor formation. Development 130, 3027–3038.

Knust, E., Bossinger, E., 2002. Composition and formation of intercellular junctions in epithelial cells. Science 298, 1955–1959.

Koshiba-Takeuchi, K., Takeuchi, J.K., Mataumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashik, N., Nakamura, H., Yasuda, K., Ogura, T., 2000. Tbx5 and the retinotectum projection. Science 287, 134–137.

Kraus, F., Haenig, B., Kispert, A., 2001. Cloning and expression analysis of the mouse T-box gene tbx20. Mech. Dev. 100, 87–91.

Lamolet, B., Pulichino, A.M., Lamonerie, T., Gauthier, Y., Brue, T., Enjalbert, A., Drouin, J., 2001. A pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. Cell 104, 849–859.

Lee, Y.S., Carthew, R.W., 2003. Making a better RNAi vector for *Drosophila*: use of intron spacers. Methods 30, 322–329.

Li, Q.Y., Newbury-Ecob, R.A., Terrett, J.A., Wilson, D.I., Curtis, A.R.J., Yi, C.H., Gebuhr, T., Bullen, P.J., Robson, S.C., Strachan, T., Bonnet, D.,

Lyonnet, S., Young, I.D., Raeburn, A., Buckler, A.J., Law, D.J., Brook, J.D., 1997. Holt–Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. Nat. Genet. 15, 21–29.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B.M., Schulz, R.A., Olson, E.N., 1995. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. Science 267, 688–693.

Lindsay, E.A., Vitelli, F., Su, H., Morishima, M., Huynh, T., Pramparo, T., Jurecic, V., Ogunrinu, G., Sutherland, H.F., Scambler, P.J., Bradley, A., Baldini, A., 2001. Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice. Nature 410, 97–101.

Lio, A., Koide, M., Hidaka, K., Morisaki, T., 2001. Expression pattern of novel chick T-box gene, Tbx20. Dev. Genes Evol. 211, 559–562.

Lo, P.C., Frasch, M., 2001. A role for the COUP-TF-related gene seven-up in the diversification of myocardial cell identities in the dorsal vessel of *Drosophila*. Mech. Dev. 104, 49–60.

Lockwood, W.K., Bodmer, R., 2002. The patterns of wingless, decapentaplegic, and tinman position the *Drosophila* heart. Mech. Dev. 114, 13–26.

Lockwood, W.K., Liu, M., Su, M.-T., Bodmer, R., 2001. A genetic model for cardiac pattern formation and cell fate determination. In: Haddad, G., Xu, T. (Eds.), Genetic models in cardiorespiratory biology. Lung biology series. Marcel Dekker Inc., New York, pp. 179–201.

Logan, M., Tabin, C.J., 1999. Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. Science 283, 1736–1739.

Meins, M., Henderson, D.J., Bhattasharya, S.S., Sowden, J.C., 2000. Characterisation of the human TBX20 gene, a new member of the T-box gene family closely related to the *Drosophila* H15 gene. Genomics 67, 317–332.

Michele, D.E., Campbell, K.P., 2003. Dystrophin–glycoprotein complex: post-translational processing and dystroglycan function. J. Biol. Chem. 278, 15457–15460.

Myat, M.M., Andrew, D.J., 2002. Epithelial tube morphology is determined by the polarized growth and delivery of apical membrane. Cell 111, 879–891.

Papaioannou, V.E., 2001. T-box genes in development: from hydra to humans. Int. Rev. Cytol. 207, 1–70.

Park, M., Wu, X., Golden, K., Bodmer, R., 1996. The *wingless* signaling pathway is directly involved in *Drosophila* heart development. Dev. Biol. 177, 104–116.

Perrin, L., Monier, B., Ponzielli, R., Astier, M., Semeriva, M., 2004. *Drosophila* cardiac tube organogenesis requires multiple phases of Hox activity. Dev. Biol. 272, 419–431.

Petronczki, M., Knoblich, J.A., 2001. DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. Nat. Cell Biol. 3, 43–49.

Plageman, T.F., Yutzey, K.E., 2004. Differential expression and function of Tbx5 and Tbx20 in cardiac development. J. Biol. Chem. 279, 19026–19034.

Poeck, B., Hofbauer, A., Pflugfelder, G.O., 1993. Expression of the *Drosophila* optomotor-blind gene transcript in neuronal and glial cells of the developing nervous system. Development 117, 1017–1029.

Porsch, M., Hofmeyer, K., Bausenwein, B.S., Grimm, S., Weber, B.H., Miassod, R., Pflugfelder, G.O., 1998. Isolation of a *Drosophila* T-box gene closely related to human TBX1. Gene 212, 237–248.

Ranganayakulu, G., Elliott, D.A., Harvey, R.P., Olson, E.N., 1998. Divergent roles for NK-2 class homeobox genes in cardiogenesis in flies and mice. Development 125, 3037–3048.

Reim, I., Lee, H.H., Frasch, M., 2003. The T-box-encoding Dorsocross genes function in amnioserosa development and the patterning of the dorsolateral germ band downstream of Dpp. Development 130, 3187–3204.

Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K., Engels, W.R., 1988. A stable genomic source of P element transposase in *Drosophila melanogaster*. Genetics 118, 461–470.

Ryan, K., Chin, A.J., 2003. T-box genes and cardiac development. Birth Defects Res., Part C Embryo Today 69, 25–37.

Singer, J.B., Harbecke, R., Kusch, T., Reuter, R., Lengyel, J.A., 1996. *Drosophila brachyenteron* regulates gene activity and morphogenesis in the gut. Development 122, 3707–3718.

Stennard, F.A., Costa, M.W., Elliott, D.A., Rankin, S., Haast, S.J., Lai, D., McDonald, L.P., Niederreither, K., Dolle, P., Bruneau, B.G., Zorn, A.M., Harvey, R.P., 2003. Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. Dev. Biol. 262, 206–224.

Su, M.-T., Fujioka, M., Goto, T., Bodmer, R., 1999. The *Drosophila* homeobox genes *zfh-1* and *even-skipped* are required for cardiac-specific differentiation of a numb-dependent lineage decision. Development 126, 3241–3251.

Szeto, D.P., Griffin, K.J., Kimelman, D., 2002. *HrT* is required for cardiovascular development in zebrafish. Development 129, 5093–5101.

Takeuchi, J.K., Ohgi, M., Koshiba-Takeuchi, K., Shiratori, H., Sakaki, I., Ogura, K., Saijoh, Y., Ogura, T., 2003. Tbx5 specifies the left/right ventricles and ventricular septum position during cardiogenesis. Development 130, 5953–5964.

Tsubota, S., Schedl, P., 1986. Hybrid dysgenesis-induced revertants of insertions at the 5′ end of the rudimentary gene in *Drosophila melanogaster*: transposon-induced control mutations. Genetics 114, 165–182.

Venkatesh, T.V., Park, M., Ocorr, K., Nemaceck, J., Golden, K., Wemple, M., Bodmer, R., 2000. Cardiac enhancer activity of the homeobox gene *tinman* depends on CREB consensus binding sites in *Drosophila*. Genesis 26, 55–66.

Ward, E.J., Skeath, J.B., 2000. Characterization of a novel subset of cardiac cells and their progenitors in the *Drosophila embryos*. Development 127, 4959–4969.

Wodarz, A., Hinz, U., Engelbert, M., Knust, E., 1995. Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. Cell 82, 67–76.

Wu, X., Golden, K., Bodmer, R., 1995. Heart development in *Drosophila* requires the segment polarity gene wingless. Dev. Biol. 169, 619–628.

Xanthos, J.B., Kofron, M., Wylie, C., Heasman, J., 2001. Maternal VegT is the initiator of a molecular network specifying endoderm in *Xenopus laevis*. Development 128, 167–180.

Xu, H., Morishima, M., Wylie, J.N., Schwartz, R.J., Bruneau, B.G., Lindsay, E.A., Baldini, A., 2004. Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. Development 131, 3217–3227.

Zaffran, S., Frasch, M., 2002. Early signals in cardiac development. Circ. Res. 91, 457–469.

Zarnescu, D.C., Thomas, G.H., 1999. Apical spectrin is essential for epithelial morphogenesis but not apicobasal polarity in *Drosophila*. J. Cell Biol. 146, 1075–1081.